# Enhanced production of calcium citrate by a 2-deoxy-D-glucose resistant mutant strain of *Aspergillus niger* using two factorial design

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**Abstract:** Over hundred isolates of the filamentous fungus *Aspergillus niger* were examined for calcium citrate productivity under submerged culture conditions in 1-L Erlenmeyer flasks. The isolate IIB-46 was selected for improvement through ultraviolet (UV) light-induced mutagenesis. Among viable mutants, strain 19 exhibited the best citrate productivity, and it was subjected to ethyl methane sulphonate (EMS) treatment. The mutant was cultured overnight and plated on 2-deoxy-D-glucose-PDA medium. A mutant strain EMS-6 gave the highest citrate production (86.48 g/l) which varied significantly ($p \leq 0.05$) from that of the wild type. The optimal citrate synthase activity (28 U/ml/min) was achieved at the optimal fermentation conditions with 26.5 g/l DCM. Maximum glucose consumption was found to be 130 g/l (over 90 % substrate conversion rate). Citrate productivity was increased over 28 % when the process parameters, incubation period (72 h), initial pH (6.5), glucose as carbon source (15 %), inoculum size ($1.875 \times 10^6$ CFU/ml) and corn steep liquor (CSL) as nitrogen source (0.5 %) were optimized using a 2-factorial Plackett-Burman design. The model terms were highly significant (*HS*) thus suggesting the potential commercial utility of the mutant (df=3~0.0218).

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**Keywords:** *Aspergillus niger*; Calcium citrate fermentation; Mutagenesis; 2-Factorial design; 2-deoxy-D-glucose

## 1. Introduction

Calcium citrate finds extensive applications in the pharmaceutical industry, and research is on-going to increase the production of this salt to meet its commercial demand (Bayraktar and Mehmetoglu, 2001). Citrate salts are produced by three different methods, chemical oxidation of glucose with hypo-chlorite, electrolytic oxidation of glucose solution containing a measured quantity of bromide and a fermentation process where specific microorganisms are grown in a medium containing glucose and other necessary ingredients (Hanok, 2009). Strains of *Aspergillus niger* have higher capability of producing calcium citrate than other microorganisms (Tripathi et al., 1999). Citrate yield has been improved by the use of chemicals such as methyl methane sulfonate (MMS), Ethyl methane sulphonate (EMS) or nitrous acid (NA). The amount of microbial calcium citrate produced from glucose in submerged culture is dependent on such factors as type and concentration of the carbon source, incubation period, pH, type and size of inoculum (Das, 1973; Nagata et al., 1998).

The main objective of the present study was to isolate and select a novel mutant of *A. niger* from different local habitats or by induced mutagenesis, capable of producing a large amount of calcium citrate in the culture medium. The metabolite 2dg was used to stabilize the culture against back mutation. The 2-factorial Plackett-Burman experimental design was used to further identify the significant variables influencing calcium citrate hyper production from the selected mutant culture.

## 2. Material and Methods

### 2.1. Isolation of organism

*Aspergillus niger* isolates were obtained from soil samples collected from different localities of Lahore (Pakistan) by the serial dilution method (Clark et al., 1958) and maintained on potato dextrose agar (PDA), pH 5.6 medium. The cultures, morphologically identified after Koneman et al. (1991), showed the following characteristics: colonies on PDA spread rapidly, mycelium was bright yellow and produced dark brown conidial heads, conidial heads were globose with an average diameter of 550 µm, phialids having chains of conidia were born on the metullae, which were fairly uniform, conidia were globose and were 2.5-10 µm diameter. Initial colonies were transferred to PDA slants. The slant cultures were incubated at 30±2ºC for 3-5 day until maximal sporulation occurred and stored at 4ºC in a cold-cabinet (P342, Griffin, England).

### 2.2. Inoculum preparation

A conidial suspension from an agar slant of a 4-6 day old culture of *A. niger* was prepared by adding 10 ml of sterile diacetyl ester of sodium sulpho succinic acid (monoxal OT) to the slant. The resulting suspension was aseptically transferred to a sterile MacConkey bottle containing silica gel beads

(~1.5 mm diameter) and shaken vigorously to break the clumps of conidia. The number of conidia in the inoculum was determined with the help of a Thomas Counting Chamber (M05, Wallenberg, Switzerland).

**2.3. Culture media**

Glucose salt-$CaCO_3$ medium containing (g/l): Glucose, 150; $(NH_4)_2SO_4$, 2.5; $MgSO_4.7H_2O$, 0.5; $KH_2PO_4$, 1; and $CaCO_3$, 32; was used as medium 1 (M1) for calcium citrate productivity. In medium 2 (M2), glucose was replaced by corn starch, and liquefaction was carried out by mixing it with 20 ml of 1 N HCl. Starch slurry (20 %) was prepared in distilled water and the pH was adjusted to 2 with 0.1 N NaOH/HCl. The mixture was autoclaved for 30 min at 12 psi, cooled and neutralized with $CaCO_3$ followed by filtration through a glass sintered filter. For medium 3 (M3), sugarcane molasses (50 %, w/v sugars) was used in place of glucose or corn starch. The medium was clarified according to the method of Panda et al. (1984). The sugar content of cane-molasses ranged from 350-450 g/l, which was diluted to about 250 g/l. Thirty-five millilitre of 0.1 N $H_2SO_4$ was added into 1-L of molasses and placed in a water bath at 90±2°C for about 1 h. After cooling at room temperature, the medium was neutralized with lime (CaO) and left to stand overnight. The clear supernatant was diluted so as to contain 15 % (w/v) total reducing sugars. Nutrient salts were then added and dissolved to obtain a homogenous mixture.

**2.4. Fermentation procedure and significant process parameters**

Calcium citrate productivity was accomplished by submerged culture in 1-L cotton-wool plugged Erlenmeyer flasks containing 200 ml of sterilized glucose salt-$CaCO_3$ medium (M1 optimized). The medium was inoculated by transferring 1 ml of conidial suspension ($1.25\times10^6$ CFU/ml) under aseptic conditions. The flasks were placed in a rotary shaker (GFL 544, Gallenkamp, UK) for incubation at 30±2°C with an agitation speed of 200 rpm for 72 h. The effect of various nitrogen sources such as urea, corn steep liquor (CSL), peptone, yeast extract and beef extract was also investigated. Among these, CSL was found to be the best; therefore its concentration was varied from 0.5 to 2 %. All the experiments were run parallel in a set of triplicates.

**2.5. Culture improvement through induced mutagenesis**

**2.5.1. UV irradiation**

One millilitre of a conidial suspension was exposed to UV radiations for different time intervals (5-150 min) under a UV lamp (λ=253 nm at 50 cycles/s, 220 V, Mineral Light UVS.12, California, USA). The dose given to the conidial suspension was $1.2\times10^2$ J/m$^2$/s. Approximately 0.5 ml of the irradiated suspension was poured onto a medium containing malt extract 2 %, agar 2 %, bromocresol green dye 1 % and pH 4.8. The plates were incubated at 30±2ºC and calcium citrate productivity was compared with that of conidia that were not exposed to UV light. The initial colonies were selected on the basis of bright yellow zones produced due to citrate hydrolysis of bromocresol dye. The mutants were selected from the plates having at least 90 % death rate.

**2.5.2. Chemical mutation**

A volume of 50 ml of Vogel's medium containing (g/l): trisodium citrate, 2.5; $NH_4NO_3$, 2; $KH_2PO_4$, 5; $(NH_4)_2SO_4$, 4; $MgSO_4.7H_2O$, 0.2; peptone, 2; and yeast extract, 1; at pH 5.5 was added to a 250-ml conical flask. Twelve to 15 chromic acid washed marble chips (2.5 mm, diameter) were dispensed in the medium to break up mycelial pellets. The flask was sterilized at 15 psi (121°C) for 15 min. Then 2 ml of sterilized 50 % (w/v) glucose stock solution was aseptically added into the autoclaved Vogel's medium as an additional carbon source. The flask was inoculated with 1 ml of conidial suspension of the selected UV mutant. The inoculum was allowed to grow at 30°C in a rotary shaking incubator for 24 h. The mycelial cells were harvested by centrifugation at 8332×g for 15 min, washed twice and re-suspended in saline (0.085 % yeast extract, 0.5 % NaCl, pH 7.2). Approximately 5 ml of the vegetative mycelial suspension was aseptically transferred to a sterile centrifuge tube for subsequent mutagenesis.

EMS was prepared at concentrations of 0.5, 1, 1.5, 2, 2.5 and 3 mg/ml using phosphate buffer (pH 7.2). Five mL of each EMS solution was added to 5 ml of the suspension. After various time intervals (between 30-60 min), the tubes were centrifuged at 6800×*g* for 20 min. The cells were washed twice by centrifugation (centrifuge refrigerated, D-37520, Osterodeam-Harz, Germany) with phosphate buffer to remove the remaining traces of the chemical mutagen. Afterwards the volume in the tubes was raised to 5 ml with sterilized distilled water. Approximately 0.1 ml of each dilution was transferred to the individual Petri plates containing PDA oxgall bromocresol green medium, pH 4.8. After 72 h of incubation at 30ºC, the number of colonies on each plate was counted with the help of a colony counter (GM-16, Tibetan, Germany). Colonies were selected on the basis of yellow zones produced due to citrate hydrolysis of bromocresol dye. Mutant strains of *A. niger* were selected in comparison with the control (run parallel but without EMS treatment) and transferred to PDA slopes. The cultures were incubated at 30ºC for 4-6 days.

2.6. Induction of resistance in *A. niger* against 2dg:

Potential mutant strains were sub-cultured overnight in nutrient mineral-salt medium (M3), harvested during the exponential phase of growth ($1.35\times10^7$ CFU/ml), washed with sterilized distilled water and plated on 2dg-PDA medium. The level of 2dg was varied from 0.005 to 0.035 µg/ml. Glucose was used instead of sucrose because its hydrolysis by the mutant culture resulted in the accumulation of some reducing sugars other than glucose (Ray and Banik, 1999). Colonies appearing between 4-6 days were cultured again and those exhibiting vigorous growth were tested for calcium citrate productivity in shake flasks. Samples were drawn periodically, washed and plated on PDA medium to select the strains insensitive against 2dg. Higher 2dg levels ranging from 0.075 to 0.1 µg/ml were also tried to find found the exact concentration of the stabilizing agent. The master mutant culture was preserved in a glass MacConkey bottle under sterile paraffin oil at –20ºC.

**2.7. Analytical methods**

**2.7.1. Dry cell mass (DCM)**

The fermented broth was centrifuged at 4560×*g* for 10 min and the mycelia were transferred to a pre-weighed Whatman filter paper No. 44. These were washed thoroughly with 0.05 N HCl to remove adhering materials and dried at 105°C, overnight.

**2.7.2. Sugar concentration**

The dinitro salicylic acid (DNS) method was used for glucose estimation (Miller, 1959). The % $T_{575nm}$ was measured using a UV/VIS spectrophotometer (Hitachi U-2000, Japan).

**2.7.3. Calcium citrate productivity**

Calcium citrate present in the supernatant sample was determined by disodium ethylene diamine tetra acetic acid (dEDTA) titration. The fermented broth was centrifuged at 8832×g for 15 min. To an accurately measured volume of fermented broth sample (1 ml), 2 ml of 3 N HCl was added and distilled water was added to a final volume of 150 ml while stirring constantly. Approximately 20 ml of 0.05 M dEDTA was added using a burette. Then 15 ml of 1 N NaOH and 300 mg of hydroxynapthol blue indicator were added and titration was continued to a blue end point. The % yield of calcium citrate was determined on the basis of glucose consumed.

**2.7.4. Citrate synthase activity**

The enzyme activity was measured after Koneman et al. (1991). Approximately 0.5 ml of the sample was added to two separate glass test tubes of 24×150 size (one for test purpose and one as a control). One drop of 2 % sodium azide was also added. The tubes were shaken thoroughly and placed in a water bath at 30ºC. Glucose (10 %, 4.5 ml) was added in 1.1 M acetate buffer, pH 5.6, to the test sample. $H_2SO_4$ (4.5 ml of 5 N) was added to each test sample while 4.5 ml glucose to each of the control. Then 2 ml of freshly prepared 5 % KI along with 1 drop of 1 % ammonium molybdate was added to each tube and allowed to stand for 3 min. The test samples were titrated against 0.02 N $Na_2S_2O_3$ using starch as an indicator.

**2.8. Determination of kinetic parameters**

For determining the kinetic parameters of batch fermentation process for calcium citrate productivity, the procedure of Pirt (1975) was adopted.

**2.9. Application of Plackett-Burman experimental design**

The variables affecting improved citrate productivity were identified using the 2-factorial system i.e., Plackett-Burman experimental design (Ahuja et al., 2004). The variables were denoted at two widely spaced intervals and the effect of individual parameters on calcium gluocnate productivity was calculated by the following equations,

$$E_o = (\Sigma M_+ - \Sigma M_-) / N \qquad I$$

$$E = \beta_1 + \Sigma\, \beta_2 + \Sigma\, \beta_3 + \beta_{123} \qquad II$$

In Eq. I, $E_o$ is the effect of first parameter under study while M+ and M– are responses of citrate productivity by the mutant strain of *A. niger* EMS-6. N is the total number of optimizations. In Eq. II, E is the significant parameter, $\beta_1$ is the linear coefficient, $\beta_2$ the quadratic coefficient while $\beta_3$ is the interaction coefficient among significant process parameters.

2.9. Statistical analysis**:**

Duncan's multiple range tests (Spss-16, version 9.8) were applied under one-way analysis of variance (I-ANOVA) and the treatment effects were compared according to Snedecor and Cochran (1980). Significance was presented in the form of probability ($p\leq0.05$) values.

**3. Results**

In the present study, over one hundred different strains of *Aspergillus niger* were isolated and tested for calcium citrate productivity in Erlenmeyer flasks. *A. niger* IIB-46 was the best producer of calcium citrate and hence treated with UV irradiation for different intervals of times. Mutant *A. niger* UV-19, isolated after 90 min of UV irradiation, exhibited maximal calcium citrate productivity. The complete death of organism was observed after 120 min of UV irradiation. To obtain the best producer strain of *A. niger,* the UV selected mutant (UV-19) was subjected to EMS treatment for 30-60 min for further improvement in terms of citrate productivity. A total of 31 EMS mutants were isolated based on the size of zones formed due to

citrate hydrolysis of bromocresol dye in the plates. EMS-6 gave the highest calcium citrate productivity (86.48 g/l) and differed significantly ($p \leq 0.05$) from the wild type and other mutants. A dose of 2.5 mg/ml EMS given for 45 min was found to be optimal for the enhancement in calcium citrate productivity. The death rate was found to be ~99 %. The complete death of organism was observed 60 min after EMS treatment. Mutant strain *A. niger* EMS-6 was stabilized after treating at various 2dg levels.

The time course studies showed that calcium citrate production was maximal 72 h after conidial inoculation by the mutant strain *A. niger* EMS-6 (Figure 1). The DCM was found to be 22 g/L. Further incubation did not increase citrate productivity.

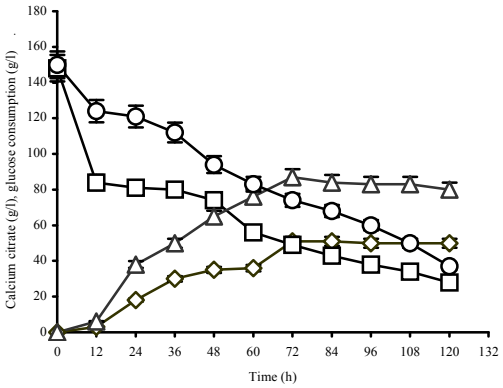



Figure 1. Rate of calcium citrate production by wild and mutant strains of *A. niger*. Glucose added 150 g/L, temperature 30°C, pH 6. Y bars indicate the standard error from the mean value (-◊- Calcium citrate by parent, -Δ- Calcium citrate by mutant, -□- Glucose consumption by parent, -○- Glucose consumption by mutant).

In the present studies, three culture media were tested for calcium citrate productivity in shake flasks (Figure 2). Of these, M1 supported maximal calcium citrate productivity and varied significantly ($p \leq 0.05$) compared to other media.

In the present study, at an initial pH of 5 or less, both mycelial growth and subsequent calcium citrate productivity remained low, while at an initial pH of 6.5, the consumption of glucose as well as product formation was improved significantly. However, a further increase in the initial pH other than the optimal reduced the rate of bioconversion of glucose to calcium citrate (Figure 3).

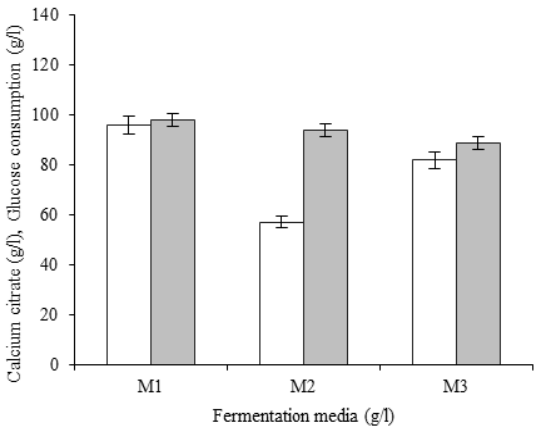



Figure 2. Evaluation of different fermentation media for calcium citrate productivity by mutant strain of *A. niger* EMS-6. Temperature 30°C, pH 6.5, incubation period 72 h. Y bars indicate the standard error from the mean value (-□- Calcium citrate productivity, -■- Glucose consumption).

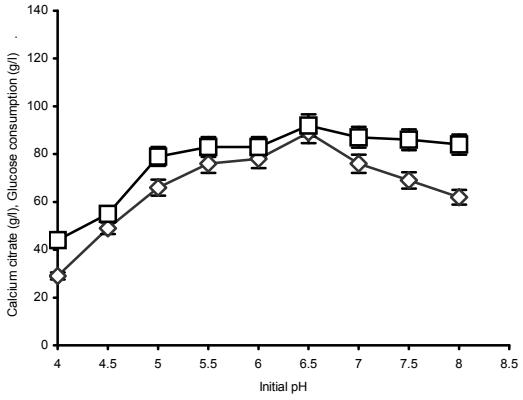



Figure 3. Effect of initial pH on calcium citrate productivity by mutant strain of *A. niger* EMS-6. Glucose added 150 g/l, temperature 30°C, incubation period 72 h. Y bars indicate the standard error from the mean value (-◊- Calcium citrate productivity, -□- Glucose consumption).

Calcium citrate productivity increased with the increase in glucose concentration and was found optimal at 15 % (w/v) level. Further increase in glucose concentration resulted into a lower glucose utilisation and hence calcium citrate formation (Figure 4).

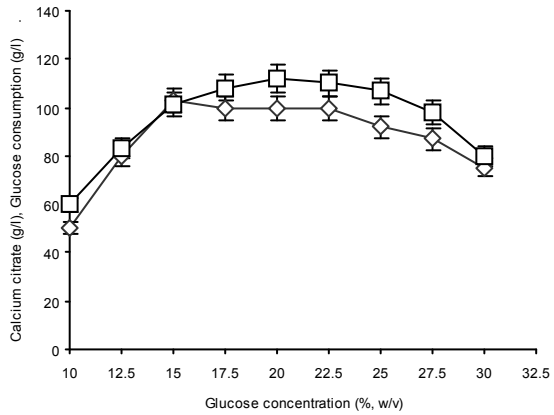



Figure 4. Effect of glucose concentration on calcium citrate productivity by mutant strain of *A. niger* EMS-6. Initial pH 6.5, temperature 30°C, incubation period 72 h. Y bars indicate the standard error from the mean value (-◊- Calcium citrate productivity, -□- Glucose consumption).

The productivity was maximal when 1.5 ml of conidial suspension ($1.875\times10^6$ CFU/ml) was used as an inoculum (Figure 5). It was also found that further increase in the size of inoculum resulted in a higher level of mycelial growth but a lower level of calcium citrate formation. Selection of nitrogen source is very essential for calcium citrate productivity.

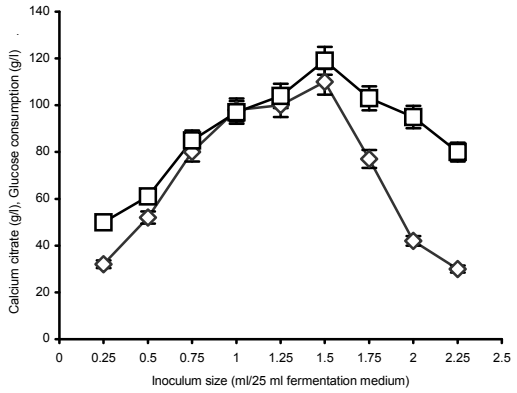
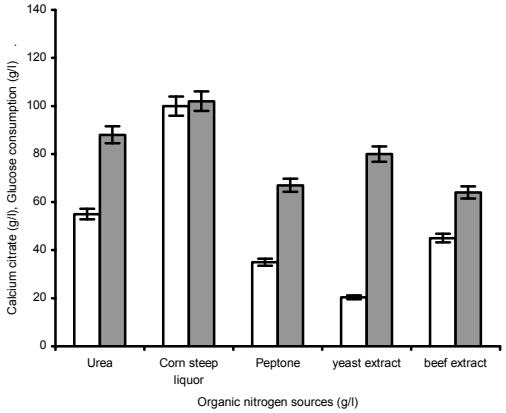
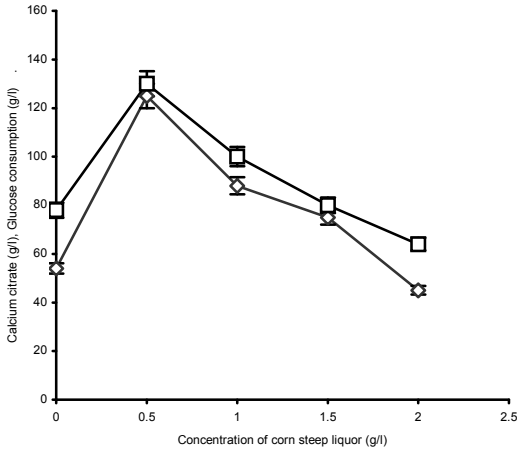



Figure 5. Effect of inoculum size on calcium citrate productivity by mutant strain of *A. niger* EMS-6. Glucose added 150 g/l, temperature 30°C, incubation period 72 h. Y bars indicate the standard error from the mean value (-◊- Calcium citrate productivity, -□- Glucose consumption).

Of the organic nitrogen sources tested, corn steep liquor (CSL) was found to enhance calcium citrate productivity (Figure 6).




a) Different organic nitrogen sources




b) Different conc. of CSL

Figure 6. Effect of organic nitrogen sources on calcium citrate productivity by mutant *A. niger* EMS-6. Temperature 30°C, pH 6.5, glucose added 150 g/l. Y bars indicate the standard error from mean value (-□- Calcium citrate productivity, -■- Glucose consumption); (-◊- Calcium citrate productivity, -□- Glucose consumption).

The 2-factorial experimental system i.e., Plackett-Burman design was applied to determine the significant process parameters involved in calcium citrate productivity by the mutant strain of *A. niger* EMS-6 (Table 1). The validation of the model was investigated under the conditions predicted against the responses obtained for improved calcium citrate productivity. A differential correlation was observed between the observed and predicted values. The optimal levels of the parameters for improved

calcium citrate productivity in shaking culture were incubation period (72 h), initial pH (6.5), glucose concentration (15 %), inoculum size (6 %, v/v) and CSL (0.5 %, w/v). The optimal level of DCM was found to be 26.5 g/l.

Table 1: Application of 2-factorial design at various process parameters for enzyme activity, DCM formation and calcium citrate productivity by the mutant strain of *A. niger* EMS-6*

| Process parameters at 2-factorial design | | | | | Calcium citrate productivity (g/l) | |
|---|---|---|---|---|---|---|
| Time period (h)[A] | Glucose (g/l)[B] | Initial pH[C] | Inoculum size (h)[D] | CSL (0.5 %)[E] | Observed | Predicted |
| 48 | 10 | 5 | 4 | na | 24 | 34 |
| 60 | 12.5 | 5.5 | 4 | na | 32 | 45 |
| 72 | 15 | 6 | 6 | 0.5 | 95 | 122 |
| 72 | 15 | 6.5 | 6 | 0.5 | 113 | 128 |
| 84 | 17.5 | 7 | 8 | 1 | 78 | 85 |

*The different letters represent significant process parameters for calcium citrate productivity. Statistical analysis of the model was based on 2-factorial experimental design. 'na' means that CSL was not added.

The statistical analyses of the responses for calcium citrate productivity were also performed (Table 2). The correlation (0.245*E*+0005), A, B, C and D for E values depicted that the model was highly significant ($p \leq 0.05$). The analysis of linear, quadratic and interaction coefficients were performed on the fermentative results.

Table 2: Statistical analysis of 2-factorial design at various process parameters for calcium citrate productivity by the mutant strain of *A. niger* EMS-6*

| Significant process parameters | Sum mean values | F-value | Degree of freedom (df) | Probability < p > |
|---|---|---|---|---|
| A | 32.28 | 1.36 | 1 | 0.0476 |
| B | 17.24 | 12.48 | 1 | 0.0464 |
| C | 7.92 | 13.42 | 2 | 0.0385 |
| D | 0.316*E*+0005 | 18.58 | 3 | 0.0352 |
| E | 104.64 | 12.52 | 3 | 0.0218 |
| Correlation | 0.245*E*+0005 | | | |

*The letters represent significant process parameters (incubation period, sugar level, initial pH, age for inoculum development, thermophilic behaviour of the fungal mutant culture) for calcium citrate productivity. CM – 19.24; $R^2$ – 0.238.

**4. Discussions**

A successful fermentation process depends both on an appropriate strain and optimization of cultural conditions. In the present study, EMS-6 gave the highest calcium citrate productivity (86.48 g/l) and differed significantly ($p \leq 0.05$) from the wild type and other mutants. Initially high yielding isolates were obtained at 0.025 g/ml 2dg; however, their phenotype became unstable in approximately 4 weeks due to the development of resistance in the mould cells after a few generations that permitted some unstable mutants to thrive (Rossi and Ribaldi, 1996). To mitigate this problem, mutant isolates were grown on PDA medium containing higher 2dg levels. The concentration of 0.085 g/ml was found optimal, as at this level strain EMS-6 exhibited steady calcium citrate productivity.

Citrate production was found maximal 72 h after conidial inoculation by the mutant EMS-6. Further incubation did not increase citrate productivity, however glucose consumption continued to rise which resulted in the excessive growth of mold mycelia, depletion of essential nutrients and productivity of other toxic inhibitory by products like tertiary and quaternary metabolites in the culture broth. It was also hypothesized that at a longer incubation period other than the optimal, the mycelial cells reached to a maximal cell density that was limited by oxygen transfer rate which in turn reduced enzyme expression. The results revealed that relatively better product biosynthesis initiated 24 h after inoculation as there was almost a negligible productivity observed prior to this period. Hameed and Qadeer (2011) obtained the maximum amount of calcium citrate by *A. niger*, 96 h after inoculation under similar cultural conditions. Hence, the present finding was more encouraging because reduction in the time period potentially reduces the cost of product formation. M1 supported maximal calcium citrate productivity which was possibly due to the presence of glucose in this medium, which supported optimal fungal growth resulting into a better productivity of calcium citrate in the culture broth. Similar findings have also been reported by previous workers (Zhao and Xiaolong, 1999; Yang et al., 2007).

At an initial pH of 5 or less, both mycelial growth and subsequent calcium citrate productivity remained low, while at an initial pH of 6.5, the consumption of glucose as well as product formation was improved significantly. The reason may be that at pH 6.5, the mycelia produced maximal enzyme citrate synthase (28 U/ml/min activity); however, a further increase in the initial pH other than the optimal reduced the rate of bioconversion of glucose to calcium citrate. These results are in accordance with the work reported previously by Sheu et al. (2002) and Munk and Hanus (2005). At a lower pH, enzyme was almost not produced; its formation was, however started at a pH value higher than 5 and declined markedly at the neutral pH.

Increase in calcium citrate yield at 150 g/l of glucose was due to fact that at this concentration, enzyme activity in the mutant *A. niger* EMS-6 (or better oxygen supply) was optimal, which resulted in a higher yield of calcium citrate (Kundu and Das, 1985; Papagianni et al., 2003). Calcium citrate

productivity was maximal when 1.5 ml of conidial suspension ($1.875\times10^6$ CFU/ml) was used as an inoculum. It was also found that further increase in the size of inoculum resulted in a higher level of mycelial growth but a lower level of calcium citrate formation. This was due to an excessive growth of *A. niger* mycelia that resulted in the increased glucose consumption rate, thereby decreasing product formation. Corn steep liquor (CSL) was found to enhance calcium citrate productivity in the present investigation. However under similar nutritional studies, Gardener et al. (1991) optimized ammonium sulphate for the manufacture of pharmaceutical grade calcium citrate in shaking culture.

The validation of 2-factorial experimental system i.e., Plackett-Burman design was investigated under the conditions predicted against the responses obtained for improved calcium citrate productivity. A differential correlation was observed between the observed and predicted values as reported by Burkert et al. (2006). The correlation (0.245*E*+0005), A, B, C and D for E values depicted that the model was highly significant ($p\leq0.05$). Correspondingly, the lower probability values indicated that the model terms were significant. The analysis of linear, quadratic and interaction coefficients were performed on the fermentative results which highlighted that calcium citrate productivity was a function of the independent parameters (Hameed and Qadeer, 2011). The addition of CSL (0.5 %) and conidial inoculum of *A. niger* (degree of freedom 3) were found necessary for maintaining the possible spatial conformation of enzyme and thus have an important physiological role in the calcium citrate productivity. According to these results, the mutant strain of *A. niger* EMS-6 was considered as an organism of choice for calcium citrate productivity.

In conclusion, a mutant strain of *A. niger* EMS-6 was isolated after UV and EMS mutagenesis which exhibited over 5 fold higher extracellular calcium citrate productivity compared to the wild type ($p\leq0.05$). Glucose as a carbon source in the medium not only supported the fungal growth but also improved enzyme productivity (28 U/ml/min), thus enhanced product formation. The significant process parameters determined after Plackett-Burman design and the value of citrate correlation (0.245*E*+0005) depicted that the model terms were highly significant (*HS*) indicating commercial utility of the mutant culture (df 3~0.0218).

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